

# GRANULOCYTIC COLONY STIMULATING ACTIVITY IN PLASMA AND LEUKOCYTES FROM CHRONIC MYELOGENOUS LEUKEMIA PATIENTS<sup>1</sup>

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**Abstract.** Colony stimulating activity present in plasma and in leukocytes from chronic myelogenous leukemia patients and from normal volunteers was determined by double layer bone-marrow tissue culture. Plasma and leukocytes incorporated into the bottom feeder layer stimulated the formation of colonies from human bone-marrow cells cultured in the upper semi-solid agar layer. The colony stimulating activity of plasma from chronic myelogenous leukemia patients was significantly increased over that of normal plasma ( $p < .01$ ). The difference between the colony stimulating activity of chronic myelogenous leukemia and normal leukocytes was not statistically significant. After 14 days of incubation, all bone-marrow cell colonies were composed primarily of macrophages rather than granulocytes as had been previously reported.

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Colony stimulating factor (CSF), stimulates both proliferation and differentiation of granulocytic precursors *in vitro*. The stimulation obtained from this factor is referred to as colony stimulating activity (CSA). When human serum and plasma are added to murine bone-marrow cells cultured *in vitro*, the colony stimulating factor present in these substances stimulates hemopoietic stem cells in the cultured marrow to proliferate and to form colonies of up to 2000 leukocytes (Metcalf and Foster, 1967). Although human plasma and serum are capable of stimulating colony formation from murine bone-marrow cells, the stimulatory activity is not comparable to, or correlated with, the stimulation of cultured human bone-marrow cells (Lind *et al*, 1974). In fact, it has been repeatedly demonstrated that human plasma and serum are not capable of dramatically stimulating human bone-marrow colony growth. The most potent stimulus to colony formation by human bone-marrow cells has been observed with peripheral leukocytes (Robinson and Mangalik, 1972). Thus, in hu-

man bone-marrow tissue culture, human leukocyte feeder layers have been used instead of serum or plasma as a source of CSF (Robinson and Pike, 1970). The present study was undertaken to determine if the levels of colony stimulating factor in chronic myelogenous leukemia patients differed significantly from levels present in normal persons.

## MATERIALS AND METHODS

Peripheral blood from normal volunteers and from chronic myelogenous leukemia patients was collected by venipuncture using sterile 20 ml Vacutainer tubes containing two drops of preservative-free sodium heparin. After allowing cellular components of the blood samples to settle for two hours at room temperature, the plasma layer from each sample was collected and filtered through a sterile 0.22  $\mu$  Millex filter. Each leukocyte layer, along with approximately 0.5 ml of plasma, was collected into separate sterile tubes.

Leukocyte feeder layers were prepared by a modification of the procedure described by Pike and Robinson (1970). Tissue culture medium was heated to 40° and mixed 9:1 with boiled 5% Purified Agar (Difco) to give a final agar concentration of 0.5%. To this mixture human peripheral leukocytes were added to yield a final concentration of  $1 \times 10^6$  cells per ml. For the preparation of plasma feeder layers, tissue culture medium heated to 40° was mixed 9:1 with boiled 6.5% agar. Human plasma was then added 1:3 to the medium yielding a final agar concentration of 0.5%. One ml aliquots of the feeder layer mixtures were pipetted into

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35 mm plastic tissue culture plates allowed to gel at room temperature, and were stored at 37° in a humidified incubator for 1 to 5 days before addition of bone-marrow overlays.

#### Tissue Culture Medium

	ml
McCoy's 5A medium (Flow Labs)	800.0
Fetal calf serum (Gibco)	150.0
Sodium bicarbonate (7.5% sol)	6.0
Sodium pyruvate (100mM sol)	10.0
MEM Vitamin solution (100X)	4.0
MEM Amino acids (50X)	8.0
MEM Nonessential amino acids (100X)	4.0
L-Glutamine (200mM)	4.0
L-Serine (0.2mM)	0.4
L-Asparagine (0.8mM)	1.6
Penicillin/Streptomycin (Gibco #507L)	5.0
Fungazone (Gibco #529L)	5.0

Human bone-marrow was obtained from normal volunteers by aspiration from either the sternum or the iliac crest and cultured by the method of Pike and Robinson (1970) with minor modifications. Bone marrow (2 to 4 ml) was aspirated into a sterile heparinized glass syringe and allowed to settle for two hours at room temperature. Cellular components were collected, washed with tissue culture medium and centrifuged in sterile Wintrobe tubes for 10 minutes at 400 x g. The leukocyte layers were then collected and dispersed in 1 ml of tissue culture medium. Viability of the prepared cell suspension was determined using the trypan blue dye exclusion method.

In preparing bone-marrow overlays, tissue culture medium was mixed 9:1 with boiled 3% agar to yield a final agar concentration of 0.3%. Bone-marrow cells were added to this mixture to yield  $2 \times 10^5$  cells per ml. One ml aliquots of the mixture were pipetted on top of the previously prepared feeder layers. After gelation at room temperature, the plates were incubated at 37° in a humidified incubator with a constant flow of 7.5% carbon dioxide in air. Controls consisted of 1 ml aliquots of feeder-layer materials without added bone-marrow overlays and 1 ml aliquots of bone-marrow plated without feeder layers. Each sample and control was plated in triplicate.

Blood used in the preparation of normal leukocyte and plasma feeder layers was categorized as normal if the total white count, differential white count, hemoglobin concentration and hematocrit were normal. Examination and diagnosis of all chronic myelogenous leukemia patients was performed by the same hematologist in order to eliminate variation in diagnostic criteria. Diagnosis was established by bone-marrow biopsy and by the morphologic appearance of the peripheral blood and bone-marrow cells. Age of the patients ranged from 16 to 82 (median 63) with a mean age of 62. All patients were under chemotherapeutic treatment at the time of sampling.

To examine cellular differentiation and maturation within individual colonies, colonies were cut from the agar, placed on microscope slides, stained with lactic aceto-orcein or

Wright's stain, flattened with a coverslip and examined at 400X and 1000X. Colonies were counted at days 14 to 16 of incubation at 40X. Only colonies containing more than 50 cells were counted. The colony stimulating activity recorded for each sample was the mean activity observed on triplicate plates. Data are presented as the mean  $\pm$  the standard error of the mean (SEM) and were analyzed for significance by t-test and analysis of variance.

#### RESULTS

Leukocyte feeder layers prepared from 100 normal volunteers, and plasma feeder layers prepared from 31 normal volunteers, were tested for colony stimulating activity. Stimulation by normal peripheral leukocytes ranged from 0 to 90 colonies with a mean of 21 and a standard error of 1.5 colonies. Normal plasma stimulated the production of 0 to 23 colonies with a mean of  $5.3 \pm 1.1$  (figs. 1 and 2).

Leukocyte feeder layers from 29, and plasma feeder layers from 27, chronic myelogenous leukemia (CML) patients were prepared and tested for colony stimulating activity. CML leukocyte feeder layers produced 2 to 70 colonies with a mean of  $21 \pm 2.0$ . Plasma feeder layers prepared from the same patients gave rise to 0 to 30 colonies with a mean of  $9.8 \pm 1.1$  (figs. 3 and 4).

When colony stimulating activities of CML and of normal leukocyte feeder layers were compared, no significant difference was found. In contrast, colony formation by bone-marrow cells cultured on CML plasma feeder layers was significantly increased over that observed on normal plasma feeder layers ( $p < .01$ ).

No morphologic differences were noted in the colonies stimulated by normal as compared to CML leukocyte feeder layers. Small cell clusters were visible with a microscope at 40X magnification after 2 to 3 days of incubation and colony size continued to increase until days 12 to 14 when a maximum colony size of 500 to 2000 cells was reached. Two major types of colonies were seen in all cultures stimulated by leukocyte feeder layers. These consisted of tight or very compact colonies and of loose or satellite colonies. Distribution of these colony types seemed to be equal in all leukocyte-stimulated cultures examined. Colonies stimulated by both normal and CML plasma feeder

layers, although not different from each other, were smaller than those formed on normal and on CML leukocyte feeder layers and were usually compact in form. No colonies were obtained from bone-marrow cells cultured without feeder layers nor from leukocyte or plasma

feeder layers plated without bone-marrow overlays.

Colonies arising from normal leukocyte, CML leukocyte, normal plasma and CML plasma feeder layers all followed the same pattern of cellular differentiation and maturation. Initially colonies were com-

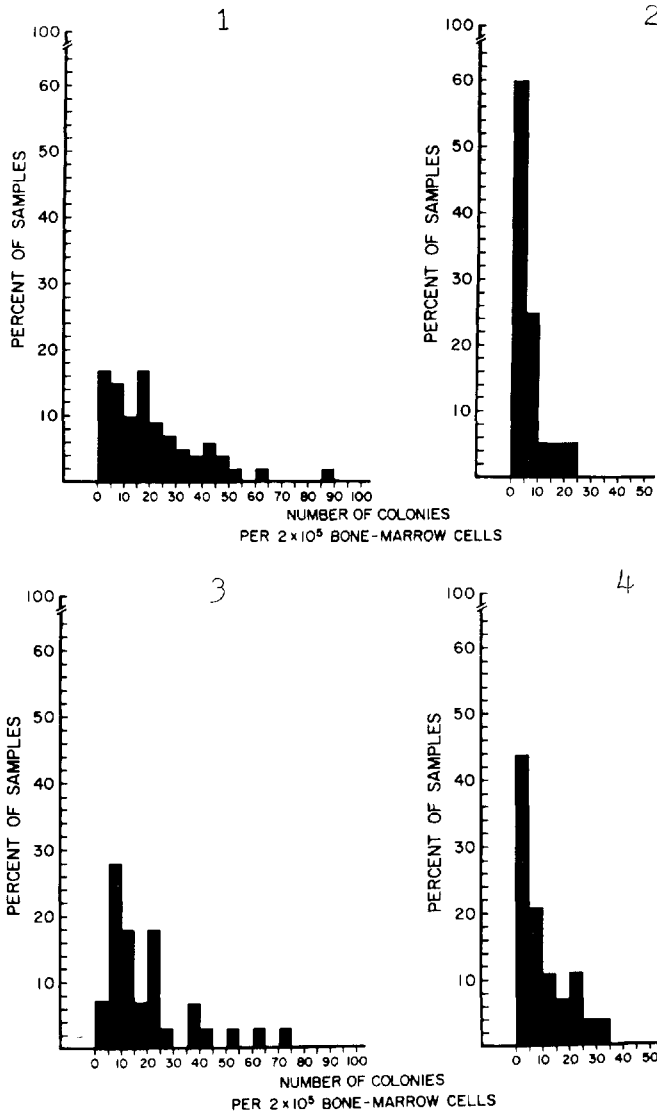


FIGURE 1. Distribution of the number of human bone-marrow cell colonies grown in triplicate on leukocyte feeder layers. Samples from 100 normal volunteers.

FIGURE 2. Distribution of the number of human bone-marrow cell colonies grown in triplicate on plasma feeder layers. Samples from 31 normal volunteers.

FIGURE 3. Distribution of the number of human bone-marrow cell colonies grown in triplicate on leukocyte feeder layers. Samples from 29 chronic myelogenous leukemia patients.

FIGURE 4. Distribution of the number of human bone-marrow cell colonies grown in triplicate on plasma feeder layers. Samples from 27 chronic myelogenous leukemia patients.

posed almost exclusively of mononuclear cells that appeared to be immature members of the granulocyte series. As cells aged, cytoplasmic granulation increased and nuclear configuration slowly changed. By day 8, approximately 80% of the cells in the colonies were morphologically similar to metamyelocytes and banded neutrophils. After day 8, a subtle granulocyte-to-macrophage transformation appeared to take place. Granulation in the cytoplasm became less prominent and the nuclei of the cells became more horseshoe-shaped and thickened. By day 14, approximately 95% of the cells in the colonies appeared to be macrophages. The remaining cells consisted of banded neutrophils and metamyelocytes.

#### DISCUSSION

The experiments described here were designed to determine if the growth and maturational characteristics of normal human bone-marrow cells induced by the colony stimulating activity (CSA) in leukocyte and in plasma feeder layers prepared from patients with chronic myelogenous leukemia (CML) differed from those found when normal leukocytes and plasma were used to stimulate colony growth. It was necessary initially to establish normal values for the CSA of normal leukocytes and of normal plasma within the tissue culture system in this laboratory. Values for the CSA of normal leukocyte and plasma feeder layers found in the literature vary dramatically and have been calculated from a limited number of samples (Mintz and Sachs, 1973; Moore *et al*, 1973a; Pike and Robinson, 1970). It was decided that a relatively large number of samples had to be tested to determine mean values for normal stimulation. Our mean values for the CSA of normal leukocyte feeder layers were based on 100 different samples, each cultured in triplicate. The mean value of the CSA of normal plasma feeder layers was based on triplicate cultures of only 31 different samples because the use of plasma as a source of CSF was not made until the sampling of normal volunteers was well underway.

Human plasma feeder layers were capable of stimulating human bone-marrow colony formation. This finding was con-

trary to previous observations which showed that human serum and plasma were capable of stimulating murine bone-marrow colony formation, but not human bone-marrow colony growth (Lind *et al*, 1974; Pike and Robinson, 1970). It was shown that murine bone-marrow cell colony formation is dependent on the agar concentration of the tissue culture medium used in the system (Bradley and Metcalf, 1966). When feeder layers are prepared, if serum or plasma is added to the agar without making a correction for the agar concentration of the media, the mixture becomes watery and does not support colony growth. In our study, when plasma feeder layers were prepared, the agar concentration of the media was always adjusted so that the final agar concentration was 0.5%.

When the colony stimulating activities of normal and of CML plasmas were compared, greater activity was found to be present in the leukemic plasma. This increased CSA in cultures stimulated by CML plasma feeder layers was statistically significant ( $p < .01$ ) and does not support the findings of Mintz and Sachs (1973) who reported CSA in serum obtained from CML patients to be similar to that in serum from normal patients.

It was not possible in this study to determine if the increase in the CSA of plasma obtained from CML patients was an innate characteristic of the disease itself, or a result of chemotherapy. Greenberg and Schrier (1974) showed that following cytotoxic drug treatment serum CSF levels were increased. Other investigators however, found the opposite to be true (Moore *et al*, 1973b). Unfortunately, in our study it was not possible to obtain samples from CML patients prior to treatment and thus this dilemma remains unsolved.

Leukocyte feeder layers prepared from CML patients, although slightly above normal, did not differ significantly from normal leukocyte feeder layer levels. This observation agrees with data presented by Moore *et al* (1973a, b) who reported below normal to normal CSA in leukocyte feeder layers prepared from treated CML patients.

After 14 days of incubation all colonies examined consisted primarily of macro-

phages. These colonies, however, developed from clones which appeared to be granulocytic earlier in the incubation period and subsequently transformed to macrophage colonies after passing through an intermediate stage in which both cell classes were present. Metcalf (1969) observed a similar colony transformation when human urine was used as a source of colony stimulating factor in murine bone-marrow tissue culture. In direct contrast is the observation of Pike and Robinson (1970) in which human bone-marrow cell colonies stimulated by human peripheral blood leukocyte feeder layers gave rise to typical mature granulocytes after 14 days of incubation.

Plasma used for the preparation of plasma feeder layers in our study was not dialyzed, thus may have contained postulated CSF inhibitors (Chan *et al*, 1971; Chan, 1971; Chan and Metcalf, 1970). This could account for the presence of predominately macrophage colonies after 14 days of incubation. Because the leukocytes used in the preparation of leukocyte feeder layers were collected in autologous plasma, it is possible that the small amount of plasma incorporated into the feeder layers along with the leukocytes contained sufficient CSF inhibitors to also cause macrophage transformation within the granulocytic colonies stimulated by these feeder layers.

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